p185^{neu}-ENCODING DNA AND THERAPEUTICAL USES THEREOF

The present invention relates to plasmid vectors containing p185^{neu} - encoding sequences and the use thereof in DNA vaccination against tumours. The plasmids according to the invention contain sequences encoding different fragments of human or rat oncoprotein p185^{neu} and are able to induce a humoral or cell-mediated immune response against tumours expressing oncogenes of the ErbB family.

The invention also relates to pharmaceutical compositions containing said plasmids and their use for the prevention or therapy of p185^{neu}-expressing tumours.

Background of the invention

Protein p185^{neu}, one of the most studied tumour antigens, has raised great interest as target for immune therapy against cancer, due to its presence on the cell membrane of some of the most common human carcinomas.

p185^{neu} is a membrane receptor encoded in the rat by proto-oncogene Her-2/neu and belonging to the family of Class I Tyrosine Kinase Receptors (RTKs), which also comprises the Epidermal Growth Factor Receptor EGF-R (ErbB-1) and other receptors related thereto (ErbB-3, ErbB-4). These receptors are involved in cell proliferation and differentiation (Hynes and Stern, 1994 BBA 1198:165) and therefore attract a great biological and clinical interest. The receptor consists of three well distinguished domains: an extracellular, transmembrane and intracytoplasmic domain. p185^{neu} is involved in the complex network of mechanisms of intracellular signal transduction and intracellular communication that regulate proliferation and differentiation processes (Boyle 1992 Curr. Op. Oncol. 4:156). Oncogene *neu* is named after the chemically-induced rat neuroglioblastoma from which it was first isolated. This activated neu form has a single point mutation that

results in the replacement of "A" with "T" and in the consequent substitution of the Valine residue at position 664 of $p185^{neu}$ with a glutamic acid residue (Val664Glu) (Bargmann et al. 1986, Cell 45:649).

Also the human *neu* homologous, ErbB-2, has been isolated and characterised and it has been demonstrated that both rat HER2/neu receptor and human ErbB2 have a significant homology with EGFR (Coussens et al. 1985, Sciente 230:1132;Yamamoto et al. 1986, Nature 319:230). While a genetic mutation in the rat sequence is the cause of constitutive receptor activation through dimerization, in ErbB-2 positive human tumours an aberrant expression of the oncogene is observed (Di Marco et al. 1990, Mol. Cell. Biol. 10: 3247; Klapper et al., 2000, Adv Cancer Res, 77:25), even though, in rare cases, activating point mutations and abnormal splicing mechanisms have been found (Kwong et al., 1998, Mol Carcinog, 23:62; Xie et al., 2000, J Natl Cancer Inst, 92:412). The overall effect is similar: gene amplification and increase in the trascription level determine an excess of $p185^{neu}$ membrane receptor, with consequent increase of active dimers intracellularly transducing growth signals in a ligand-independent manner.
The crystal structure of human and rat $p185^{neu}$ extracellular region recently reported shows that this protein is characterised by a rigid conformation that allows to interact with other ErbB receptors, without directly binding any ligands, and trigger the proliferation signal transduction (Cho HS et al. 2003, Nature 421:756).

Under normal circumstances, human $p185^{neu}$ is involved in organogenesis and epithelial growth; it is expressed at high levels during placenta formation and fetal development, whereas it is present at very low levels in adult tissues (Press et al. 1990, Oncogene 5:953).

Several studies have demostrated that human $p185^{neu}$ overexpression is associated to the neoplastic process and to the level of tumor aggression. The

overexpression of $p185^{neu}$ has been described in lung (Kern et al. 1986, Cancer Res. 50:5184), colon (Cohen et al. 1989, Oncogene 4:81), ovary (Slamon et al. 1989, Science 244:707) adenocarcinomas and in a high number of human mammary carcinomas (Slamon et al. 1989, Science 244:707; Jardines et al. 1993, Pathobiology 61:268).

The fundamental properties that make $p185^{neu}$ an optimal target for plasmid vaccination are: a) its direct involvement in cell growth and carcinogenesis, therefore clone variants that, due to tumour genetic instability, lose the expression of this antigen also lose their tumorigenicity; b) its expression on the plasmatic membrane, which makes it recognizable by antibodies even in tumour cells that lose the expression of the major histocompatibility system (Lollini P. and Forni G. 2003, Trends Immunol. 24: 62).

Studies carried out on mice transgenic for the activated rat oncogene Her-2/neu (which spontaneously develop $p185^{neu}$ positive mammary tumours) and on murine models using $p185^{neu}$ positive transplantable tumour lines, have demonstrated the possibility to prevent and cure preneoplastic lesions. As regards in particular the prevention of mammary tumours in mice transgenic for rat activated Her-2/neu, we have demonstrated that the plasmid coding for the extracellular and transmembrane domains of rat $p185^{neu}$ is able to induce an in vivo protection more effective than the plasmid encoding for the full-length rat $p185^{neu}$ or for the extracellular domain only (secreted antigen) (Amici A. et al. 2000, Gene Ther., 7: 703; Rovero S. et al. 2000, J. of Immunol., 165: 5133). Similar results have been reported by Chen et al. (1998, Cancer Res 58:1965). Other authors have demonstrated that plasmids encoding for $p185^{neu}$ - either unvaried or mutated so as to eliminate its tyrosine-kinase activity - are effective in preventing the onset of tumours following to $p185^{neu}$-positive cells inoculum (Wei WZ et al. 1999, Int.

J. Cancer 81: 748). Moreover, plasmids devoid of the signal responsible for the processing through the endoplasmic reticulum (leader), which determines cytoplasmic localization of $p185^{neu}$ antigen, proved equally effective. The protection induced by different plasmids was mainly mediated by a humoral immune response in the case of membrane expression of $p185^{neu}$, and by a T-lymphocyte-mediated immune-response in the case of cytoplasmic localization (Pilon SA et al. 2001, J. of Immunol. 167: 3201). However, combined vaccination with plasmids inducing $p185^{neu}$ overexpression in both the cytoplasm and the membrane was more effective in protecting against tumour growth (Piechocki MP et al. 2001, J. Immunol. 167: 3367).

Thus, the balance between different immune response mechanisms might be particularly important (Reilly et al., 2001, Cancer Res. 61: 880). Moreover, it has been observed that the vaccination with plasmids encoding for extracellular and transmembrane domains of rat $p185^{neu}$ is able to eradicate tumour masses with 2 mm diameter, upon inolculum of cells overexpressing $p185^{neu}$, through a number of different effector mechanisms of the immune system (T helper and T killer cells, antibodies, macrophages, neutrophiles, natural killer cells, Fc receptors, gamma interferon and perforins), which cooperate to tumor rejection (Curcio C. et al. 2003, J. Clin. Invest. 111: 1161).

Description of the invention

Various constructs encoding the human or human/rat chimeric p185 protein have been inserted in plasmid vectors and used in immunization experiments aimed at preventing tumour progression. For plasmid construction, fragments of the human $p185^{neu}$ protein containing the transmembrane domain and portions of the extracellular domain of decreasing length have been prepared from ErbB2 oncogene sequence, or portions thereof have been replaced with homologous sequences from the rat Her-2/neu cDNA so as to create chimeric plasmids.

The plasmids thereby obtained have been evaluated in vaccination experiments in mice inoculated with tumour cells overexpressing human $p185^{neu}$. Plasmids containing truncated forms of $p185^{neu}$ induced an antitumor reactivity mediated by killer and helper T lymphocytes, while chimeric plasmids induced an antibody response against both human and rat $p185^{neu}$.

Based on the results of in vivo experiments, the plasmids containing $p185^{neu}$ sequences able to induce a strong immune response of both cellular and humoral type have been selected. These plasmids, object of the present invention, contain a sequence encoding a $p185^{neu}$ fragment selected from the group consisting of SEQ ID N. 1-14 (human and rat $p185^{neu}$ reference sequences are available at Gene Bank accession numbers M11730 and X03362, respectively).

According to the invention, $p185^{neu}$ encoding sequences can be inserted in any plasmid vectors suitable for human administration. Besides the encoding sequences, the plasmids can contain functional elements for transcription control, in particular a promoter placed upstream of the encoding sequence, preferably the CMV promoter, start and stop transcription elements, selection markers, such as ampicillin or kanamicin resistance genes, CpG motifs, a polyadenilation site or transcription activators. Transcription control elements should be compatible with the use of the vector in humans. In a preferred embodiment, the plasmids of the invention contain at least 4 CpG motifs, preferably at least 8, up to a maximum of 80. The CpG motifs (ATAATCGACGTTCAA) of bacterial origin induce macrophages to secret IL-12, which in turn induce IFN gamma secretion by natural killer cells, thus activating a T helper lymphocyte-mediated response (Chu R.S. et al. 1997, J. Exp. Med., 186: 1623). Therefore, the insertion of CpG motifs in plasmid sequences enhances the immune response.

In a further embodiment, the invention provides a pharmaceutical

composition containing one or more different plasmids as defined above in association with pharmaceutically acceptable vehicles and excipients. The pharmaceutical compositions, in a form suitable for parenteral administration, preferably in the form of injectable solution, are conveniently used for DNA vaccination. Principles and methods for DNA vaccination are known to the skilled in the art and are diclosed, for example, in Liu MA 2003; J Int Med 253: 402.

In another embodiment, the invention provides a combined preparation containing at least two, preferably at least four, more preferably at least eight different plasmids for simultaneous, sequential or separate administration to a subject or patient.

Plasmids, compositions and preparations according to the invention are used in preventive or therapeutical treatment of subjects at risk of developing $p185^{neu}$-positive tumours, or patients with primary tumours, metastasis or relapses of $p185^{neu}$-positive tumours. Prevention can be primary, when the tumour is not manifest, secondary, when the tumour is in the initial phases as a preneoplastic lesion, o tertiary, in the case of tumour relapse or metastatic process. Tumours that can benefit from treatment with the plasmids of the invention are those of epithelial origin, in particular pulmonary, ovary and mammary adenocarcinomas and, more generally, tumours expressing the $p185^{neu}$ protein.

Detailed description of the invention

Construction of the plasmid backbone of pCMV3.1

To construct plasmids encoding human $p185^{neu}$ fragments and chimeric plasmids, the pCMV3.1 plasmidic backbone was used. Fragments deriving from human proto-oncogene ErbB-2 cDNA and from rat proto-oncogene Her-2/neu cDNA have been inserted in pCMV3.1 (Invitrogen, Milano, Italia) by removing with restriction enzymes DraIII (nt1531) e BsmI (nt3189) a

fragment of 1658 bp containing the replication origin f1, the replication origin and the early SV40 promoter, the gene encoding for neomicine resistance and SV40 polyadenylation signal. The resulting modified plasmid (pCMV3.1) present some advantages compared to native pcDNA3.1. In fact, the size reduction to 3900 bp and the removal of irrelevant sequences contribute to increase transfection efficacy *in vivo*.

Construction of plasmid pCMV3.1erbB2

Human ErbB2 cDNA, obtained from plasmid pSVerbB2, has been inserted in the multiple cloning site of pCMV3.1 at restriction sites HindIII and XbaI. This plasmid is used for the construction of plasmids expressing truncated $p185^{neu}$ and chimeric plasmids.

Construction of plasmids containing the sequence 4XCpG: pCMV3.1hECD-TM-4CpG and pCMV3.1hECD-TM-4noCpG

After removal of the sequence encoding the intracytoplasmic domain from plasmid pCMV3.1-erbB2, two plasmids coding for proto-oncogene ErbB2 extracellular and transmembrane regions were prepared. The procedure comprised first the restriction analysis to identify the unique sites present in the nucleotide sequence of ErbB2 cDNA. A unique site recognized by enzyme AccIII (nt 2195) about 20 bp downstream of the end of the transmembrane domain was identified.

The cytoplasmic domain was removed using the enzyme AccIII present as unique restriction site and enzyme XbaI. To re-insert at the 3'end of the DNA of the ErbB2 ECD-TM the nucleotide triplet TAA, recognized as translation stop signal, we used two synthetic sequences consisting of two sense (oligonucleotide #1, #3) and antisense (oligonucleotide #2, #4) oligonucleotides having the restrictions sites AccIII and XbaI at their ends. In these synthetic sequences there are also four repeated sequences CpG and noCpG. The latter is used as negative control. These two new plasmids have

been named pCMV3.1hECD-TM-4CpG and pCMV3.1hECD-TM-4noCpG.

Construction of the plasmids containing the sequence 8XCpG: pCMV3.1H/NhECD-TM-8CpG and pCMV3.1H/NhECD-TM-8noCpG

To add further aspecific immune stimuli we constructed a new plasmid backbone containing 4 immune-stimulating CpC sequences, called pCMV3.1 H/N-4CpG. For this purpose we modified pCMV3.1 so as to remove one of the two restriction sites for the enzyme PmeI and invert the restriction sites for HindIII and NneI present on the multiple cloning site by means of a synthetic sequence consisting of two sense (oligonucleotide #5) and antisense (oligonucleotide #6) oligonucleotides. In this new plasmid, named pCMV3.1 H/N, two synthetic sequences have been inserted, consisting of two sense (oligonucleotide #7, #9) and antisense nucleotides (oligonucleotide #8, #10), containing four repeats for the CpG and noCpG sequences in the unique restriction sites XbaI and PmeI, thus obtaining pCMV3.1 H/N-4CpG and 4noCpG. Thereafter, DNA fragments hECD-TM-4CpG and hECD-TM-4noCpG have been inserted in pCMV3.1 H/N-4CpG and in pCMV3.1 H/N-4noCpG respectively, thus obtaining two new plasmids called pCMV3.1H/N-hECD-TM-8CpG and pCMV3.1H/N-hECD-TM-8noCpG.

Construction of the plasmid containing the sequence of the second cystein domain and transmembrane domain of human $p185^{neu}$: pCMV3.1H/Nh2°cysECD-TM-8CpG

Human $p185^{neu}$ extracellular domain is characterised by two regions rich in cysteins, known as 1^{st} and 2^{nd} cystein sub-domain (1^{st} cys and 2^{nd} cys). Unlike the rat cDNA sequence containing only one site BstEII (nt1250) in the extracellular domain, located in the nucleotide region that separates 1^{st} cys from 2^{nd} cys, the cDNA sequence of the extracellular domain of ErbB2 has two restriction sites for BstEII: in addition to the site in the same position as that of rat (nt1372), a further BstEII site (nt963) is present in the portion

encoding the 1st cys of the extracellular domain. Digesting plasmid pCMV3.1H/NhECD-TM-8CpG with HindIII and BstEII, a DNA fragment consisting of the 2nd cys from the extracellular domain, the transmembrane domain, the sequence 8CpG and the plasmid pCMV3.1H/N was obtained. Then the signal for rat p185neu secretion through the endoplasmic reticulum was inserted by enzymatic DNA amplification (PCR reaction) using a sense oligonucleotide consisting of the primer T7 (oligonucleotide #11) which recognizes the T7 RNA polymerase, present at the beginning of the pCMV3.1H/N multiple cloning site, and an antisense oligonucleotide (oligonucleotide #12) having the BstEII site at its end. After purification, enzymatic digestion of the amplified fragment with restriction enzymes HindIII and BstEII and subsequent cloning, pCMV3.1H/Nh2°cys-TM-8CpG (Fig. 1) has been obtained. (Fig. 1). This plasmid was used in vaccination experiments, to have it compared with pCMV3.1 H/NhECD-TM-8CpG. Thereafter, a chimeric cDNA encoding for the fusion protein between 2nd cys and transmembrane domain (nt 1372-nt 2204) of the human sequence and 1st cys (nt 1-nt 1250) of the rat sequence has been prepared. The reconstitution of the entire protein sequence by the fusion of portions deriving from rat and human cDNAs, respectively, allows to increase the immune response.

Construction of the chimeric plasmid containing the sequence of the first cystein domain of rat p185neu and of the second cystein domain and transmembrane domain of human (nt 1-nt 1250): pCMV3.1H/N-r1°cys-h2°cysTM-8CpG

Unlike the rat cDNA sequence containing only a BstEII (nt1250) site in the extracellular domain located in the nucleotide region that separates the first and the second region rich in cysteins, the cDNA sequence of the extracellular domain of Erb2 has two restriction sites for BstEII: one in position 1372 (nt), as in the rat sequence, and the other in position 963 (nt),

i.e. in the sequence portion encoding for the 1st cys of the extracellular domain. The presence of the BstEII site in the same position both in the rat cDNA domain (1250nt) and in the human cDNA (1372nt) allowed the construction of a plasmid able to encode a fusion product between rat 1st cys and human 2nd cys. In fact, digesting pCMV3.1H/N-h2°cysTM-8CpG with restriction enzymes HindIII and BstEII allowed to replace the DNA fragment encoding for rat p185neu secretion signal with the nucleotide sequence encoding for rat 1st cys obtained through digestion of pCMV3.1rECD-TM-4CpG with the same enzymes. The product of plasmid pCMV3.1H/N-r1°cys-h2°cysTM-8CpG (Fig. 2) consists of a portion of 412 aa of rat p185neu and a portion of 274 aa of human p185neu. This new plasmid, pCMV3.1H/Nr1°cys-h2°cysTM-8CpG has been used in vaccination experiments using pCMV3.1H/N-hECD-TM-8CpG as comparative term. Surprisingly, the plasmid coding for the chimeric protein induces in mice a complete protection against tumours expressing human p185neu (Table). This protection is similar to that induced by pCMV3.1H/N-hECD-TM-8CpG. Moreover, analysis of the sera of mice vaccinated with both plasmids has evidenced a similar antibody titer towards human p185neu.

Plasmids able to encode decreasing fragments of the extracellular and trasmembrane domain of human p185 neu

Construction of seven plasmids that encode decreasing fragments of the extracellular and transmembrane domain of human p185neu, namely: pCMV3.1H/NhECD1-TM-8CpG (-70 aa), pCMV3.1H/NhECD2-TM-8CpG (-150 aa), pCMV3.1H/NhECD3-TM-8CpG (-230 aa), pCMV3.1H/NhECD4-TM-8CpG (-310 aa), pCMV3.1H/NhECD5-TM-8CpG (-390 aa), pCMV3.1H/NhECD6-TM-8CpG (-470 aa) and pCMV3.1H/NhECD7-TM-8CpG (-550 aa).

The fragment encoded by the first of these fragments is 70 aa (deletion

of 360 bp) shorter. All the others are gradually 80 aa shorter (deletions of 240 bp).

These fragments have been obtained by DNA enzymatic amplification, using seven different sense oligonucleotides with NheI restriction site (oligonucleotides #13-#19) at its end and an antisense oligonucleotide (oligonucleotide #20) able to recognise the site called "pcDNA3.1/BGH Reverse priming site" (830-850 nt) present at the 3' end of the polylinker of pCMV3.1. Further to enzymatic digestion with restriction enzymes NheI and PmeI, amplification products have been cloned in pCMV3.1H/N-neu leader, previously obtained insertng the secretion signal to the endoplasmic reticulum of rat $p185^{neu}$ in restriction sites. The DNA fragment of rat $p185^{neu}$ secretion signal has been obtained by enzymatic DNA amplification using primer T7 (oligonucleotide #11) as sense nucleotide and an antisense nucleotide (oligonucleotide #21) with NheI site at its end. The amplified fragment after purification and restriction digestions with HindIII and NheI has been cloned in plasmid pCMV3.1H/N, digested with the same enzymes, thus obtaining the pCMV3.1H/N-neu leader. Membrane expression of the different truncated forms of human $p185^{neu}$ is expected in view of the presence of the secretion signal to the endoplasmic reticulum of rat $p185^{neu}$. The plasmids encoding the truncated forms pCMV3.1H/NhECD1-TM-8CpG (Fig. 3), pCMV3.1H/NhECD2-TM-8CpG (Fig. 4), pCMV3.1H/NhECD3-TM-8CpG (Fig. 5), pCMV3.1H/NhECD4-TM-8CpG (Fig. 6) as well as the control plasmid pCMV3.1H/NhECD-TM-8CpG, protect 100% of the vaccined mice against a lethal inoculum of tumour cells expressing human $p185^{neu}$ (Table). Plasmid pCMV3.1H/NhECD5-TM-8CpG (Fig. 7) protects 60% of the animals (Table), while plasmids pCMV3.1H/NhECD6-TM-8CpG and pCMV3.1H/NhECD7-TM-8CpG (Fig. 8, 9), do not have protective effect against a lethal inoculum of tumour cells expressing human $p185^{neu}$ (Table).

The protein products expressed by the different plasmids are not secreted through the endoplasmic retuculum. The absence of consensus sequences necessary for glycosilation and for their processing through the endoplasmic reticulum, or conformational changes due to deletion of aminoacids at the -NH_2 terminus, could explain the absence of protein products in the membrane. Therefore, to further verify if the various truncated forms of the extracelllular and transmembrane domain of human $p185^{neu}$ were correctly expressed, new plasmids coding for fusion proteins characterized by epitope myc at the -NH_2 terminus were generated. These recombinant proteins are recognized by an anti-myc monoclonal antibody, therefore it is possible to analyse their expression and localisation by confocal microscopy.

First a new plasmid coding for the secretion signal to the rat endoplasmic reticulum (neu leader) and for the myc epitope has been created. Cloning has been carried out using a synthetic sequence consisting of a sense (oligonucleotide #22) and antisense (oligonucleotide #23) having at both ends the NheI site. The NheI site in the 5' position was mutated so that, once correctly ligated, it was not recognized by the enzyme. We thus obtained the pCMV3.1H/Nneuleader-myc epitope. With this plasmid, the sequences encoding human $p185^{neu}$ truncated forms have been cloned in the restriction sites NheI and PmeI. Then, 3T3 NIH fibroblasts have been transfected in vitro with plasmids using lipofectamine 2000 (Invitrogen, Milan, Italy). After 48 hours the transfected cells have been analysed with confocal microscopy, using a FITC-conjugated anti-myc monoclonal antibody (Sigma-Aldrich Srl, Milan, Italy). It has been thus demonstrated that all the pasmids-encoded truncated forms are located in the cytoplasm. 3T3 NIH fibroblasts have been transfected in parallel with plasmid pCMV3.1H/NhECD-TM-8CpG and analysed with confocal microscopy using c-erbB2/c-neu Ab-3 monoclonal antibody (Oncogene, Boston, MA) as primary antibody and a

FITC-conjugated anti-mouse secondary antibody (PharMigen, San Diego, CA). It was thus observed that human ECD-TM is expressed in the membrane. The results obtained using the first four plasmids described previously (pCMV3.1H/NhECD1-TM-8CpG, pCMV3.1H/NhECD2-TM-8CpG, pCMV3.1H/NhECD3-TM-8CpG, pCMV3.1H/NhECD4-TM-8CpG), demonstrate that a cellular response is sufficient for antitumour prevention. However, it is known that contemporaneous activation of the cellular and humoral response is necessary for a more effective therapy (Rielly et al., 2001, Cancer Res 61:880). As already described in the previous paragraph, the chimeric protein encoded by plasmid pCMV3.1H/N-r1°cys-h2°cysTM-8CpG is able to protect 100% of the vaccined animals and is able to induce a strong humoral response in the mice.

Chimeric plasmids able to encode for five different man-rat chimeric $p185^{neu}$

For the construction of plasmids coding for chimeric proteins, we selected pCMV3.1H/NhECD1-TM-8CpG, pCMV3.1H/NhECD2-TM-8CpG, pCMV3.1H/NhECD3-TM-8CpG and pCMV3.1H/NhECD4-TM-8CpG. These four plasmids protect 100% of the vaccinated mice against a lethal inoculum of tumour cells expressing human $p185^{neu}$. Also plasmid pCMV3.1H/NhECD5-TM-8CpG has been selected, even if it protects only 60% of the vaccinated mice, because the encoded protein differs only by 17 aa from that encoded by pCMV3.1H/Nh2°cysECD-TM-8CpG (275 aa), which protects 20% of the vaccinated mice. We can hypothesize that the peptide sequence of 17 aa corresponds to an important epitope for the induction of an effective immune response.

DNA fragments encoding for rat $p185^{neu}$ portions have been obtained by DNA enzymatic amplification. To amplify these cDNA fragments six oligonucleotides having all the same orientation, namely that of T7 primer

(oligonucleotide #11), have been used, while the five antisense have been designed to recognize rat cDNA in the proper positions and have the restriction site for NheI at their ends (oligonucleotides #24-#28). After purification and digestion with restriction enzimes HindIII and NheI, the amplified fragments have been inserted in the corresponding plasmids (pCMV3.1H/NhECD1-TM-8CpG, pCMV3.1H/NhECD2-TM-8CpG, pCMV3.1H/NhECD3-TM-8CpG, pCMV3.1H/NhECD4-TM-8CpG pCMV3.1H/NhECD5-TM-8CpG) and digested with the same restriction enzymes. In this way we obtained five new plasmids able to code for chimeric proteins of 689 aa, of which 2 (Val-Ser) belong to restriction site NheI used for the conjuction between rat and human DNA. The presence of these two aa renders both human and rat portions heteroclytic.

The chimeric proteins differ for human $p185^{neu}$ decreasing portions and rat $p185^{neu}$ increasing portions. Plasmid pCMV3.1H/Nr73-hECD1-TM-8CpG (Fig. 10) encodes 73 aa of the rat $p185^{neu}$ extracellular domain and 614 aa of human $p185^{neu}$. Plasmid pCMV3.1H/Nr153-hECD2-TM-8CpG (Fig. 11) encodes 153 aa of the rat $p185^{neu}$ extracellular domain and 534 aa of human $p185^{neu}$. Plasmid pCMV3.1H/Nr233-hECD3-TM-8CpG (Fig. 12) encodes 233 aa of the rat $p185^{neu}$ extracellular domain and 454 aa of human $p185^{neu}$. Plasmid pCMV3.1H/Nr313-hECD4-TM-8CpG (Fig. 13) encodes 313 aa of the rat $p185^{neu}$ extracellular domain and 374 aa of human $p185^{neu}$. Plasmid pCMV3.1H/Nr393-hECD5-TM-8CpG (Fig. 14) encodes 393 aa of the rat $p185^{neu}$ extracellular domain and 294 aa of human $p185^{neu}$. Indirect evidence of the membrane expression of human/rat chimeric $p185^{neu}$ encoded by these plasmids has been obtained immunizing mice with the five new plasmids and with pCMV3.1H/N-r1°cys-h2°cysTM-8CpG as positive control. The sera of all vaccinated mice contain specific antibodies against human $p185^{neu}$. Moreover, the animals vaccinated with plasmids encoding the five different

chimeric proteins are also protected with a lethal inoculum of tumour cells expressing human $p185^{neu}$.

EXAMPLES

Example 1 - Construction of plasmid pCMV3.1H/N-r1°cys-h2°cysTM-8CpG

To construct chimeric plasmid pCMV3.1H/N-r1°cys-h2°cysTM-8CpG we started from plasmid pCMV-ECD-TM, which expresses the extracellular and transmembrane domain of rat $p185^{neu}$ (Amici et al 2000, Gene Ther., 7: 703). pCMV-ECD-TM was digested with restriction enzymes HindIII and XbaI (BioLabs, Beverly, MA) to separate the insert from the plasmid backbone.

Restriction digestion with enzymeHindIII:

plasmid DNA (1 μg/μl)	10 μl
restriction buffer 10X (NEB2)	10 μl
HindIII (10U/μl)	5 μl
H_2O	75 μl
	100 μl final volume

The mixture was incubated at 37°C for 4 hours and the digestion product controlled by electrophoresis on 1% agarose gel using a molecular weight marker and undigested plasmid as control.

Once confirmed plasmid linearization, DNA was precipitated by adding 1/10 volume of 3 M sodium acetate at pH 5.2 and 2 volumes of cold absolute ethanol.

The sample was kept on ice for 20 min., then centrifugated with a minicentrifuge at 14.000 rpm for 12 min. The pellet was washed three times with 1 ml 70% cold ethanol, dried under vacuum for 5 min, then resuspended in 84 μl H_2O and enzymatically digested with restriction enzyme XbaI.

Restriction digestion with enzyme XbaI:

DNA resuspended in H_2O (10 µg)	84 µl
Restriction buffer 10X (NEB2)	10 µl
BSA 100X (100mg/ml)	1 µl
XbaI (10U/ml)	5 µl
	100 µl

The mixture was incubated at 37°C for 4 hours and the digestion product was precipitated and dried as described above. DNA was resuspended in 30 µl H_2O.

The two DNA fragments corresponding to the plasmid backbone (pCMV of 4400bp) and to the insert (ECD-TM of 2100bp) were separated by electrophoresis on a 1% agarose gel.

The band corresponding to the insert was removed and DNA eluted from the gel using a Qiaquick gel extraction kit (Qiagen Italy).

In parallel, the new plasmid backbone (pCMV3.1H/N–4CpG) wherein the DNA fragment corresponding to rat p185 ECD-TM, was digested with the same restriction enzymes and eluted on agarose gel.

The DNA fragments corresponding to rat ECD-TM and the linearized plasmid pCMV3.1H/N-4CpG were used to obtain pCMV3.1H/N-rECD-TM-4CpG by ligation reaction.

Ligation reaction

DNA insert (rECD-TM) (50 ng/µl)	2 µl
Linearized plasmid DNA (pCMV3.1H/N4CpG) (50 ng/µl)	1 µl
Reaction buffer 10X for T4 DNA ligase	1 µl
T4 DNA ligase (2U/µl)	1 µl
H_2O	5 µl
	10 µl

The ligation reaction was incubated at 16°C for 4 hours.

The ligation product was then used to transform the *E. coli* bacterial strain DH5 α. The bacterial cells have been made competent with the $CaCl_2$ technique.

Transformation of the bacterial strain DH5 α:

Competent bacterial cells	100 µl
Ligation product	5 µl

To make the plasmid DNA penetrate the competent cells, these were kept on ice for 40 min. and submitted to thermal shock (1.5 min. at 42°C and then 2 min. on ice).

After adding 1 ml LB growth medium, the transformed bacterial cells were incubated at 37°C for 1 hour to restore their physiological conditions.

The cell suspension was then centrifuged at 6000 rpm for 1 min. and the pellet was resuspended in 100 µl LB.

The cells were seeded in Petri dishes containing selective solid medium (LB with agar + ampicillin 100 µg/ml) and grown at 37°C for 1 night. Ampicillin allows the growth of cells containing plasmid pCMV3.1H/N-rECD-TM-4CpG which confers ampicillin-resistance.

The resulting clones were analysed by alkaline lysis to select those containing the recombinant plasmid pCMV3.1H/N-rECD-TM-4CpG.

To obtain chimeric plasmid pCMV3.1H/N-r1°cys-h2°cysTM-8CpG, plasmid pCMV3.1H/N-rECD-TM-4CpG was digested with restriction enzymes BstEII and XbaI to remove the second cystein domain together with the transmembrane domain of rat $p185^{neu}$. At the same time, plamid pCMV3.1hECD-TM-4CpG was digested with the same enzymes to isolate the DNA fragment corresponding to the second cystein subdomain and transmembrane domain of the human gene.

Digestion with BstE11:

plasmid DNA (1 μg/μl)	10 μl
Restriction buffer 10X (NEB3)	10 μl
BstEII (10U/μl)	5 μl
H_2O	75 μl
	100 μl final volume

The mixture was incubated at 60°C for 4 hours.

Restriction digestion with XbaI, recovery of the fragments to be used for cloning, ligation reaction and transformation of competent cells have been described previously.

The resulting chimeric plasmid pCMV3.1H/N-r1°cys-h2°cysTM-8CpG has been sequenced using the automatic ABI PRISM 310 Genetic Analyzer (Applied Biosystem), to verify the correct insertion of the fragment corresponding to the 2nd cystein subdomain and the transmembrane domain of the human gene.

Example 2 - in vivo test

Animals

Balb/cAnCr (H-2^d) female mice aged about seven weeks have been used for all experiments.

The animals, supplied by Charles River Laboratories (Calco, MI, Italy), are grown in aseptic conditions and in accordance with the European Community guidelines.

Intramuscular administration followed by in vivo electroporation

To avoid unwanted contractions of the tibial muscle each mouse was anaesthetized by i.p. inoculum of 300 μl avertine, made of 0.58 g tribromoethanol (Sigma-Aldrich) and 310 μl Tert-Amyl alcohol (Aldrich) dissolved in 39.5 ml deionized H_2O. All mice have been then shaved in correspondence of the tibial muscle for the inoculum.

The animals have been vaccinated in correspondence of both antero-tibial muscles, with 40 µl of solution containing 50 µg DNA.

The DNA-containing mixture was prepared shortly before use, in conformity with the indications of Dr. F. Pericle (Valentis, Inc., The Woodlands, Texas, USA). This solution contains 1.25 mg/ml plasmid DNA, 6 mg/ml poly-L-glutamate sodium salt (Sigma-Aldrich, S.r.l., Milano, Italia), 150 mM sodium chloride (Fluka, BioChemika, Buchs, Switzerland) and distilled water free from endotoxins (Nucleare Free Water, Promega Corporation) to a final volume of 1 ml.

After about 5 min from the inoculum, the treated area was submitted to electroporation, by application of two electric impulses having an intensity of 375 V/cm^2, each lasting 25 ms, using the electroporator Electro Square Porator (T820, BTX, San Diego, CA, USA). The transcutaneous electric impulses have been applied by use of two square steel electrodes placed at 3 mm from each other, beside each paw. Gene immunization by electroporation was carried out twice for each animal 21 and 7 days before inoculum of tumour cells.

Inoculum of tumour cells

The mice have been inoculated with a suspension containing 2 x 10^5 D2F2/E2 cells. These cells derive from a mammary tumour spontaneously generated in a hyperplastic alveolar node of a BALB/c mouse and express high levels of human p185.

In vivo evaluation of tumour growth

Tumour growth was evaluated weekly by palpation and the dimensions of the tumours were measured along two perpendicular diameters with a calibre. Neoplastic masses measuring more than 3 mm are considered as tumours.

Tumour growth was followed for 100 days from tumour inoculum or

until the tumour had grown to a diameter higher than 10 mm, then animals were sacrificed.

Table

Mice: female BALB/c

Tumour: D2F2-E2 espressing human $p185^{neu}$

plasmids	Number of mice	protection	antibodies
pCMV3.1H/N-8CpG	5	0%	-
pCMV3.1H/N-hECD-TM-8CpG	5	100%	+++
pCMV3.1H/N-hECD1-TM-8CpG	5	100%	-
pCMV3.1H/N- hECD2-TM-8CpG	5	100%	-
pCMV3.1H/N- hECD3-TM-8CpG	5	100%	+
pCMV3.1H/N- hECD4-TM-8CpG	5	100%	++
pCMV3.1H/N- hECD5-TM-8CpG	5	60%	-
pCMV3.1H/N- hECD6-TM-8CpG	5	0%	-
pCMV3.1H/N- hECD7-TM-8CpG	5	0%	-
pCMV3.1H/N-r1°cys-h2°cys.-TM-8CpG	5	100%	+++

List of oligonucleotides synthesized and used for plasmid construction

#1. AccIII-TAA-4CpG-erbB2 sense 71 nt

5'CCGGAAGTAAATAATCGACGTTCAAATAATCGACGTTCAAATAATCGACGTTCAAATAATCGACGTTCAAT 3'

#2. XbaI-TAA-4CpG-erbB2 antisense 71 nt

5'CTAGATTGAACGTCGATTATTTGAACGTCGATTATTTGAACG TCGATTATTTGAACGTCGATTATTTACTT 3'

#3. AccIII-TAA-4noCpG-erbB2 sense 71 nt

5'CCGGAAGTAAATAATAGAGCTTCAAATAATAGAGCTTCAAA TAATAGAGCTTCAAATAATAGAGCTTCAAT 3'

#4. XbaI-TAA-4noCpG-erbB2 antisense 71 nt

5'CTAGATTGAAGCTCTATTATTTGAAGCTCTATTATTTGAAGCT CTATTATTTGAAGCTCTATTATTTACTT 3'

#5. HindIII-NheI sense 27nt

5' CTAGGAAGCTTGTTTAACTTGCTAGCT 3'

#6. HindIII-NheI antisense 27 nt

5'AGCTAGCTAGCAAGTTAAACAAGCTTC 3'

#7. XbaI-4CpG-neu sense 68 nt

5'CTAGATAATCGACGTTCAAATAATCGACGTTCAAATAATCGA CGTTCAAATAATCGACGTTCAAGTTT 3'

#8. PmeI-CpG-neu antisense 64 nt

5'AAACTTGAACGTCGATTATTTGAACGTCGATTATTTGAAC GT CGATTATTTGAACGTCGATTAT 3'

#9. XbaI-4noCpG-neu sense 68 nt

5'CTAGATAATAGAGCTTCAAATAATAGAGCTTCAAATAATAG AGCTTCAAATAATAGAGCTTCAAGTTT 3'

#10. PmeI-4noCpG-neu antisense 64 nt

5'AAACTTGAAGCTCTATTATTTGAAGCTCTATTATTTGAAGCT CTATTATTTGAAGCTCTATTAT 3'

#11. T7 primer

5'TAATACGACTCACTATAGGG 3'

#12. BstEII-neuleader antisense 32 nt

5'GGCCGGTTACCCGCGATTCCGGGGGGCAGGAG 3'

#13. hECD1-TM-sense-NheI 35 nt

5'CCGGCTAGCTAGCCTGTCCTTCCTGCAGGATATCC 3'

#14. hECD2-TM-sense-NheI 35 nt

5'CCGGCTAGCTAGCGGAGGGGTCTTGATCCAGCGGA 3'

#15. hECD3-TM-sense-NheI 35 nt

5'CCGGCTAGCTAGCCTGCCCACTGACTGCTGCCATG 3'

#16. hECD4-TM-sense-NheI 35 nt

5'CCGGCTAGCTAGCTGCACCCTCGTCTGCCCCCTGC 3'

#17. hECD5-TM-sense-NheI 35 nt

5'CCGGCTAGCTAGCCCGCTCCAGCCAGAGCAGCTCC 3'

#18. hECD6-TM-sense-NheI 35 nt

5'CCGGCTAGCTAGCAACACCCACCTCTGCTTCGTGC 3'

#19. hECD7-TM-sense-NheI 35 nt

CCGGCTAGCTAGCCCCAGGGAGTATGTGAATGCCA 3'

#20. pcDNA3.1/BGH Reverse primer 20 nt

5'TAGAAGGCACAGTCGAGGCT 3'

#21. NheI-neuleader-antisense 43 nt

5'CCGGCTAGCTAGCCGCGATTCCGGGGGGCAGGAGGGCGAGG AG 3'

#22. His-myc-sense-noNheI 69 nt

5'CTAGGCATCATCATCATCATCATAATGGTCATACCGGTGAAC AAAAACTCATCTCAGAAGAGGATCTGG 3'

#23. His-myc-antisense-NheI 69 nt

5'CTAGCCAGATCCTCTTCTGAGATGAGTTTTTGTTCACCGGTAT GACCATTATGATGATGATGATGATGC 3'

#24. NheI-73neu antisense 35 nt

5'CCGGCTAGCTAGCGCTGGCATTGGCAGGCACGTAG 3'

#25. NheI-153neu antisense 35 nt

5'CCGGCTAGCTAGCCAGGATCTCTGTGAGACTTCGA 3'

#26. NheI-233neu antisense35 nt

5'CCGGCTAGCTAGCGCCCTTGCACCGGGCACAACCA 3'

#27. NheI-313neu antisense35 nt

5'CCGGCTAGCTAGCTCCCACTTCCGTAGACAGGTAG 3'

#28. NheI-393neu antisense 35 nt

5'CCGGCTAGCTAGCAATGCCGGAGGAGGGGTCCCCA 3'